CIRCULAR DICHROIC SPECTRUM OF THE LACCASE-PEROXIDE DERIVATIVE

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1. Introduction

The mechanism of dioxygen reduction to water by the blue copper oxidases is an intriguing biochemical problem [1–5]. Much effort has recently been invested in the search of possible intermediates in this reaction [4–7]. Laccase, the blue oxidase extracted from the Japanese lacquer tree (*Rhus Vernicifera*) is one of the best characterized among this group of enzymes and we were able to show that it forms a specific, high affinity and stable derivative upon reacting with H_2O_2 [4,5]. On chemical grounds the formation of a peroxy complex as an intermediate in the reaction of fully or partially reduced laccase with O_2 is expected. However, only recently has evidence been found showing marked spectral similarity between the derivative formed on reacting the fully oxidized enzyme with H_2O_2 and the intermediates observed for the reaction of O_2 with laccase reduced to different extents or during the steady state of the catalytic cycle ([15] and O. F., M. G. and I. P., in preparation). The formation of the peroxy derivative is revealed as a specific increase in the extinction maximum at ~325 nm. Yet no change in the EPR spectrum of the enzyme could be resolved even at liquid helium temperature. The type 3 copper pair of laccase has been proposed as the site of interaction with peroxide (and most probably also with O_2) in laccase [5,8].

In this report we present further evidence for the affinity and specificity of interaction between H_2O_2 and laccase from CD measurements. The formation of the peroxy-laccase derivative is shown to be accompanied by a prominent CD change in the 260–360 nm range. Smaller changes are also exhibited throughout the visible region. These spectral features confirm the nature of the laccase peroxy species as analogous to the oxygenated hemocyanines and of oxy-tyrosinase. Changes in the visible absorption spectra of laccase upon peroxide or F^- binding may reflect interactions between the type 2 and type 3 sites. On the basis of tracer and EPR studies [6,7], these sites were proposed to be in close proximity. Fluoride ions were found to modulate the near ultraviolet absorption spectrum both of native-oxidized and of peroxy-laccase. These effects may also be rationalized in terms of the spatial proximity and possibly direct interactions between the sites.

Abbreviations: CD, circular dichroism; EPR, electron paramagnetic resonance; kK, cm^{-1}; PPB, potassium phosphate buffer

2. Materials and methods

Laccase was prepared from acetone powder of the lacquer of *Rhus Vernicifera* according to a modified version of the procedure in [5,9]. The spectroscopic, EPR, and catalytic properties of the enzyme were similar to those in [4,9]. The ratio between A_{280} and A_{614} was 15.2. The protein concentration was determined from the absorbance at 614 nm [10] ($\epsilon = 5700$ $M^{-1}cm^{-1}$). Laccase solutions were made up in 0.1 M potassium phosphate buffer (pH 7.0, $I = 0.22$ M, doubly distilled water). The protein concentrations used extended from 5×10^{-6} M to 2×10^{-4} M. All chemicals were of analytical grade and used without further purification.

Absorption spectra were recorded on a Zeiss DMR

10 spectrophotometer and CD spectra on a Roussel-Jouan Dichrographe III. All spectra were measured in 1 cm cuvettes at 25°C over the 1000–250 nm spectral range (10–40 kK).

3. Results

The addition of an equimolar amount of hydrogen peroxide to native, oxidized laccase results in changes both of absorption and CD spectrum. The differences in the absorption spectra of the native enzyme were found to extend into the visible region resulting in $\Delta\epsilon_{615} \approx -185 \pm 40\ M^{-1}cm^{-1}$ (fig.1). The effect of H_2O_2 on F^- treated laccase is different from that found for the native enzyme; the shape of the near ultraviolet band is slightly altered while its intensity enhancement is the same (fig.1). The CD changes extend from 250–1000 nm and are exceptionally prominent around 330 nm where a new negative band is resolved (fig.2). At 275 nm a marked increase in the CD is observed, significantly without any change in the well-resolved band at 294 nm (fig.3).

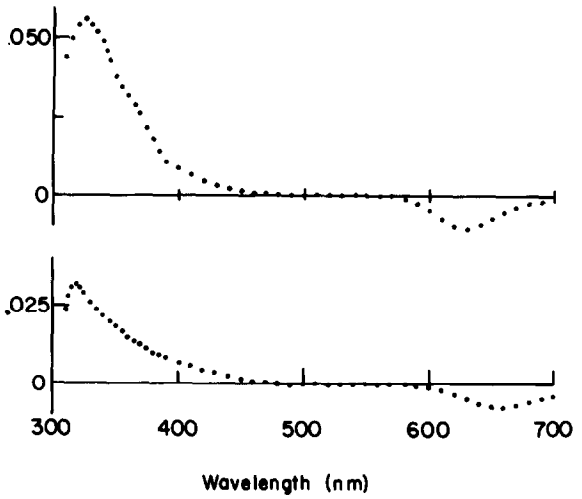

Fig.1. The interaction of oxidized laccase with H_2O_2. (upper) Calculated difference spectrum of native-oxidized laccase reacted with equimolar H_2O_2 minus native-oxidized laccase. Enzyme, 6.6×10^{-5} M. $T = 22$°C. Ordinate: absorbance. (lower) Calculated difference spectrum of laccase–fluoride complex reacted with equimolar H_2O_2 minus laccase–fluoride complex. Laccase, 3.8×10^{-5} M, was preincubated with 0.1 M NaF for several hours. Ordinate: absorbance.

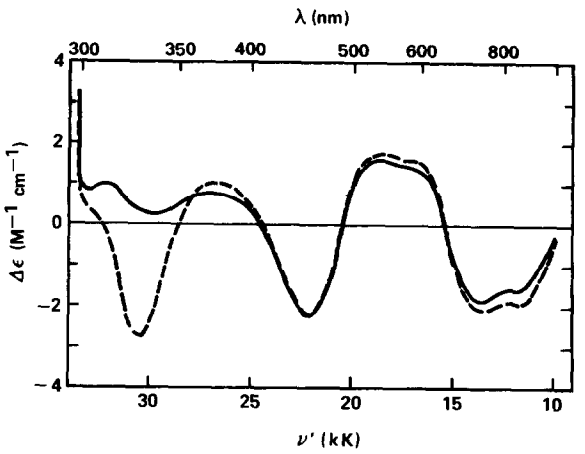

Fig.2. CD spectra of laccase in the near ultraviolet and visible regions. (–) A native laccase, 1.2×10^{-4} M in 0.1 M PPB. $T = 25$°C. (- -) Same laccase solution with 1.3×10^{-4} M H_2O_2 added.

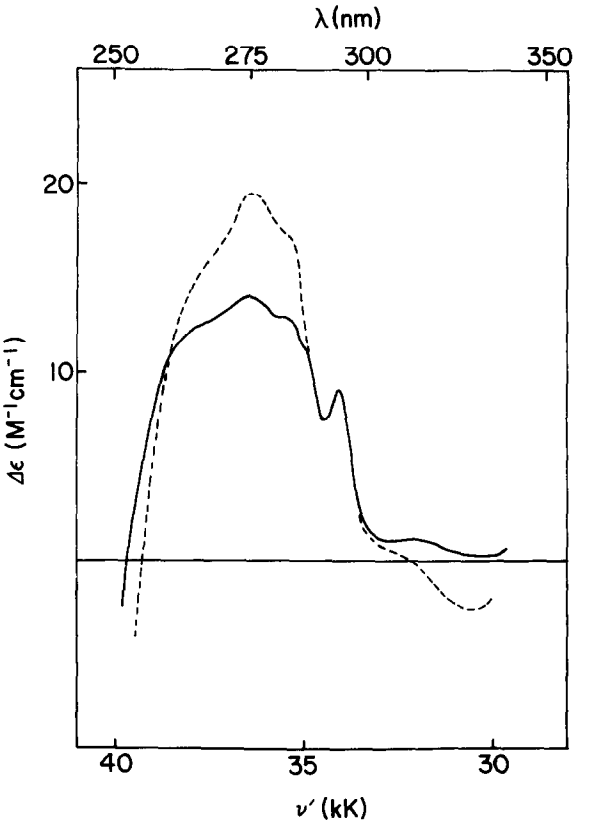

Fig.3. CD spectra of laccase in the ultraviolet region. (–) Native laccase, 5.8×10^{-6} M in 0.1 M PPB. $T = 25$°C. (- -) Same laccase solution with 1.1×10^{-5} M H_2O_2 added.

The formation of the new CD spectrum occurs in <10 min when both reactants are at 0.1 mM (fig.2). This spectrum remains stable for more than 6 h without noticeable changes. Even when both laccase and H_2O_2 are decreased to μM levels the reaction proceeds to completion in <20 min (fig.3).

4. Discussion

The formation of a new spectral species upon reacting oxidized laccase with hydrogen peroxide was found to be characterized both by an absolute specificity and a high affinity for H_2O_2 since the reaction was complete even at μM levels of the reagents [4]. As no changes in the EPR spectrum were found to accompany the optical-spectral changes, the site involved in the interaction with H_2O_2 was proposed to be the type 3 copper ion pair. The CD spectra reported here constitute unambiguous evidence for the formation of a new species and lend further support to the above assignment. This point was not as clear in the absorption difference spectrum because of the overlap with the original type 3 copper band. The resemblance between the near ultraviolet absorption band of laccase-peroxide and bands characteristic of oxy-hemocyanin and oxy-tyrosinase [11–13] was interpreted as an indication [4] for a close structural relationship between the respective sites of laccase and of the two other copper proteins. Furthermore a striking similarity exists between the near ultraviolet CD band of the laccase-peroxide presented here and the near ultraviolet CD band reported for oxy-hemocyanins [14]. Compelling evidence has been presented from Raman spectroscopy that in oxy-hemocyanin, oxygen is bound as a peroxide, bridging the two Cu(II) ions [15,16].

The CD changes observed in the visible range are small yet reproducible. It is noteworthy that upon peroxide formation changes also occur in the absorbance in this region (fig.1). Ranges of change and lack of change practically coincide in both absorption and CD spectra. This may be a reflection of perturbations in the type 1 Cu(II) chromophore yet the more interesting possibility of these changes being due to the type 2 Cu(II) site should also be considered. The proposed proximity between type 3 and type 2 sites [7] makes such an effect reasonable. The modulation of the peroxide difference spectrum in the near ultraviolet by F^- ions lends further support to this notion.

The CD changes in the 250–300 nm range (fig.3) are remarkable. Particularly interesting is the large increase at 275 nm which takes place without any variation of the 294 nm band. This might be due to a change in dissymmetry in the environment of tyrosine residues of the enzyme occuring without any perturbation of the tryptophan residues. This is of interest as tryptophan fluorescence intensity was found [17] to be correlated with the redox state of type 1 copper but not with that of the type 3 site, in line with the above interpretation. However the magnitude of the change at 275 nm ($\Delta(\Delta\epsilon) = 5\ M^{-1}\ cm^{-1}$) would imply a major conformational rearrangement which is difficult to reconcile with the lack of effect on the tryptophans. Thus if this CD change does indeed stem from tyrosines it must be due to an unusual effect on the optical activity of a limited number of residues, possibly involved with the type 3 site. Alternatively this change at 275 nm may result from a different chromophore (e.g., Cu-imidazole), related to the type 3 site, induced or enhanced upon interaction with peroxide. Interestingly such CD changes were also resolved in certain hemocyanins upon oxygen binding [14].

The fact that species of remarkable similarity to the laccase-peroxide can be spectroscopically observed during the catalytic cycle of the enzyme with excess O_2 or the reduced enzyme with limiting concentrations of oxygen has been reported [5]. Recent CD measurements of intermediates formed under these conditions show great similarity to the spectra of the laccase-peroxide reported here.

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